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Induction of cutaneous graft-*versus*-host disease by local injection of unprimed T cells

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SUMMARY

The skin is a major target organ in human graft-*versus*-host disease (GVHD) after bone-marrow transplantation. GVHD can be induced in mice by i.v. injection of T cells into unirradiated semi-allogeneic or lethally irradiated allogeneic recipients. However, in the murine systemic GVHD model, cutaneous lesions occur only in lethally irradiated recipients. Since lethal irradiation itself might induce the epidermal cell damage, several investigators have employed another murine model of cutaneous GVHD, in which cutaneous lesions were induced by intradermal injection of alloreactive T cell clones. Using this system, it has been reported that both MHC class I- and II-reactive T cell clones can induce cutaneous GVHD in non-irradiated or sublethally irradiated recipients. However, it has remained unknown whether or not freshly prepared T cells are able to induce cutaneous GVHD after local injection into non-irradiated recipients. We show that unprimed T cells can induce cutaneous GVHD after local injection into unirradiated MHC class II- or I+II-disparate recipients. In contrast to alloreactive T cell clones, unprimed T cells could elicit only mild cutaneous lesions in MHC class I-disparate recipients. Since sublethal irradiation of MHC class I-disparate recipients did not result in the manifestation of cutaneous lesions after injection of unprimed T cells, host anti-donor responses by radiosensitive cells could not be responsible for this phenomenon. This experimental system provides a useful model for analysis of the regulation mechanisms in the induction of GVHD by unprimed T cells.

Keywords graft-*versus*-host disease cutaneous lesions MHC T cell subsets

INTRODUCTION

The skin is a major target organ in human graft-*versus*-host disease (GVHD) after bone-marrow transplantation together with the liver, intestine, lymphoid and haematopoietic organs (Glucksberg *et al.*, 1974). There have been several reports (Rappaport *et al.*, 1979; Jaffee & Claman, 1983; Charley *et al.*, 1983; Piquet *et al.*, 1987) describing cutaneous lesions in murine systemic GVHD. In spite of the disadvantage that lethal irradiation itself might induce the epidermal cell damage, cutaneous lesions of systemic GVHD were induced in lethally irradiated mice after reconstitution with donor type bone marrow cells, probably because it is difficult to reproduce cutaneous lesions in the murine systemic GVHD system without lethal irradiation. In fact, we have confirmed that neither gross nor microscopic cutaneous lesions were observed in murine systemic GVHD induced in unirradiated or sublethally irradiated recipients (unpublished observation). For an alternative approach to induce GVHD in the murine skin, we have demonstrated (Watanabe *et al.*, 1985, 1989) that cloned cytotoxic T cells elicited cutaneous GVHD after local injection into the skin of unirradiated or sublethally irradiated histo-incompatible mice. Similar results were also reported by others using T cell clones (Tyler *et al.*, 1984; Shiohara, Narimatsu & Nagashima, 1987). However, because T cell clones established *in vitro* have very strong cytotoxic activities and differ from normal T cells in their homing ability (Dailey, Gallatin & Weissman, 1985) and interleukin-2 (IL-2) dependency (Watanabe *et al.*, 1985), cutaneous GVHD induced by cloned T cells would differ from that by fresh T cells. Moreover, if a different clone is used, different results might be obtained.

From these viewpoints, we attempted to induce cutaneous GVHD by local injection of freshly prepared (referred to as 'unprimed') T cells into non-irradiated mice. We show that unprimed parental T cells are able to induce cutaneous GVHD following local injection into unirradiated MHC class I- and/or II-disparate F1 mice. In contrast to the previous findings using established alloreactive T cell clones, MHC class I-disparate cutaneous GVHD induced by unprimed T cells was very weak as compared with MHC class II- or I+II-disparate cutaneous GVHD. Since sublethal irradiation of MHC class I-disparate

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recipients did not result in the development of comparable cutaneous lesions, host anti-donor responses by radiosensitive cells would not be responsible for this phenomenon.

MATERIALS AND METHODS

Mice

C57BL/6 (B6); B6.*C-H-2*^{bm1} (bm1), H-2K mutant strain (Bailey, Snell & Cherry, 1971); and B6.*C-H-2*^{bm12} (bm12), I-A mutant strain (McKenzie *et al.*, 1979) were originally from the Jackson Laboratory, Bar Harbor, ME. B10.Thy-1.1, Thy-1 congenic strain (Muto *et al.*, 1983) was kindly provided by Dr T. Sado, Division of Physiology and Pathology, National Institute of Radiological Science, Chiba, Japan. These mouse strains and the F1 hybrids used in this study were bred at our animal facilities. Female mice, aged 9–15 weeks, were used throughout this study.

Monoclonal antibodies

The following monoclonal antibodies (MoAbs) were used: anti-CD4 (GK1.5 ascites fluid, diluted 1/10 for immunohistochemical study or 1/300 for cell depletion); anti-CD8 (53-6.7 ascites fluid, diluted 1/10 for immunohistochemical study; 83-12-5 culture supernatant, diluted 1/300 for cell depletion); biotinylated anti-Thy-1.1 (purchased from Meiji Institute of Health Science, Tokyo, Japan). Erythrocyte- and spleen-absorbed guinea pig serum was used as the source of complement for cell depletion.

Preparation of donor cells

Cell suspension from spleens of normal B6 or B10.Thy-1.1 mice was prepared in Eagle's MEM (Nissui Pharmaceutical Co., Tokyo, Japan) containing 5 mM HEPES (Sigma Chemical Co., St Louis, MO) and 2% fetal calf serum (FCS) (Bockneck Laboratories, Rexdale, Ontario, Canada). The cell suspension was incubated with NH_4Cl lysing buffer to remove erythrocytes and passed through a nylon wool column. The column-passed spleen cells are referred to as whole T cells. Depletion of $CD8^+$ and $CD4^+$ cells were made by treating whole T cells with each MoAb + complement. Briefly, whole T cells (1×10^7 cells/ml) were incubated with a diluted MoAb for 30 min at room temperature, then washed, and incubated with complement for 45 min at 37°C. About >85% of the cells obtained by this treatment showed the desired phenotype, and T cells with the depleted phenotype were not detected by flow cytometric analysis (data not shown). Anti-CD8 + complement-treated T cells and anti-CD4 + complement-treated T cells are referred to as $CD4^+$ and $CD8^+$ cells, respectively.

Induction of cutaneous GVHD

Donor cells were washed in medium without FCS and 1×10^7 viable cells were injected intradermally in a volume of 50 μl at the shaved back skin of (B6 × bm1)F1, (B6 × bm12)F1 or (bm1 × bm12)F1 recipient mice. B6 mice injected with syngeneic cells or with medium alone served as controls. In some experiments, recipient mice were irradiated at a dose of 4 Gy from a ^{60}Co source. Each experimental group consisted of two to three mice and the representative results of two to three independent experiments are shown. Recipient mice were killed at various intervals after injection and the skin around the cell-injection site was excised and divided into two parts. One part was fixed in 4% paraformaldehyde, dehydrated in graded alcohol and xylene, and embedded in paraffin. Sections of 4 μm thickness were cut and stained with haematoxylin and eosin. The remaining skin was snap-frozen in isopentane that had been pre-cooled in a bath of acetone and dry ice. Sections of 5 μm were cut in a cryostat, and stored at −20°C until use.

Immunoperoxidase staining procedures

Frozen sections were air-dried and fixed in cold acetone for 10 min. After incubation with normal sheep serum, endogenous avidin-binding activity was blocked using a blocking kit (Vector Laboratories, Burlingame, CA). Then, sections were allowed to react with primary MoAb for 60 min at room temperature, followed by incubation with biotinylated sheep anti-rat immunoglobulin antibody (Amersham International, Amersham, UK) diluted 1/50 with 5% normal mouse serum for 45 min and finally with horseradish-peroxidase-labelled streptavidin (Amersham) diluted 1/50 in PBS for 45 min. Between each incubation, sections were washed in PBS. Horseradish-peroxidase-binding sites were detected in 0·05% diaminobenzidine and 0·01% H_2O_2, Tris–HCl buffer containing 10 mM NaN_3.

RESULTS

Mild erythema was occasionally observed at the skin around the cell-injected site of recipient mice. However, this was not a constant finding. Unlike local cutaneous GVHD induced by cloned alloreactive T cells (Watanabe *et al.*, 1985, 1989), ulceration was not observed. Histological changes in the skin of unirradiated recipient mice are summarized in Table 1.

Histological changes in the skin of MHC class II-disparate recipient mice

When B6 whole T cells were injected into unirradiated (B6 × bm12)F1 mice, typical histological changes of cutaneous GVHD (Lerner *et al.*, 1974) were observed 4–6 days after injection (Fig. 1b). The epidermis showed thickening, and many mononuclear cells infiltrated into the intercellular spaces in the basal to spinous cell layers of the epidermis, resulting in eosinophilic epidermal cell necrosis. In some sections, peripheral aggregation of intraepidermal mononuclear cells around dyskeratotic keratinocytes (satellitosis) was observed. These histological features were essentially identical to those of cutaneous GVHD induced by alloreactive T cell clones (Shiohara *et al.*, 1987; Watanabe *et al.*, 1989). Kinetic analysis revealed that injected donor cells in the dermis decreased gradually and cutaneous lesions disappeared by 7 days after injection.

When donor cells were purified to $CD4^+$ or $CD8^+$ cells, both T cell subsets were able to induce cutaneous GVHD (Fig. 2). The histological features were essentially the same as those by whole T cell injection. When $CD8^+$ cells were injected, however, thickening of the epidermis was relatively mild.

When B6 cells were injected into syngeneic B6 recipients, mild thickening of the epidermis and a few intraepidermal mononuclear cells were occasionally observed, but neither massive infiltration of mononuclear cells nor epidermal cell

Table 1. Histological changes in the skin of recipient mice

Donor cells	Recipient	Thickening of the epidermis	Intra-epidermal infiltrating cells	Epidermal cell necrosis
B6 Whole T	(B6 × bm1)F1	+	+	+
CD4		+	+	−
CD8		−/+	+	+
B6 Whole T	(B6 × bm12)F1	++	++	++
CD4		++	++	++
CD8		+	++	++
B6 Whole T	(bm1 × bm12)F1	++	++	++
CD4		++	++	++
CD8		+	++	++
B6 Whole T	B6	+	+	−
CD4		+	+	−
CD8		−/+	+	−

Each change is graded as: −, none; +, mild; or ++, severe.

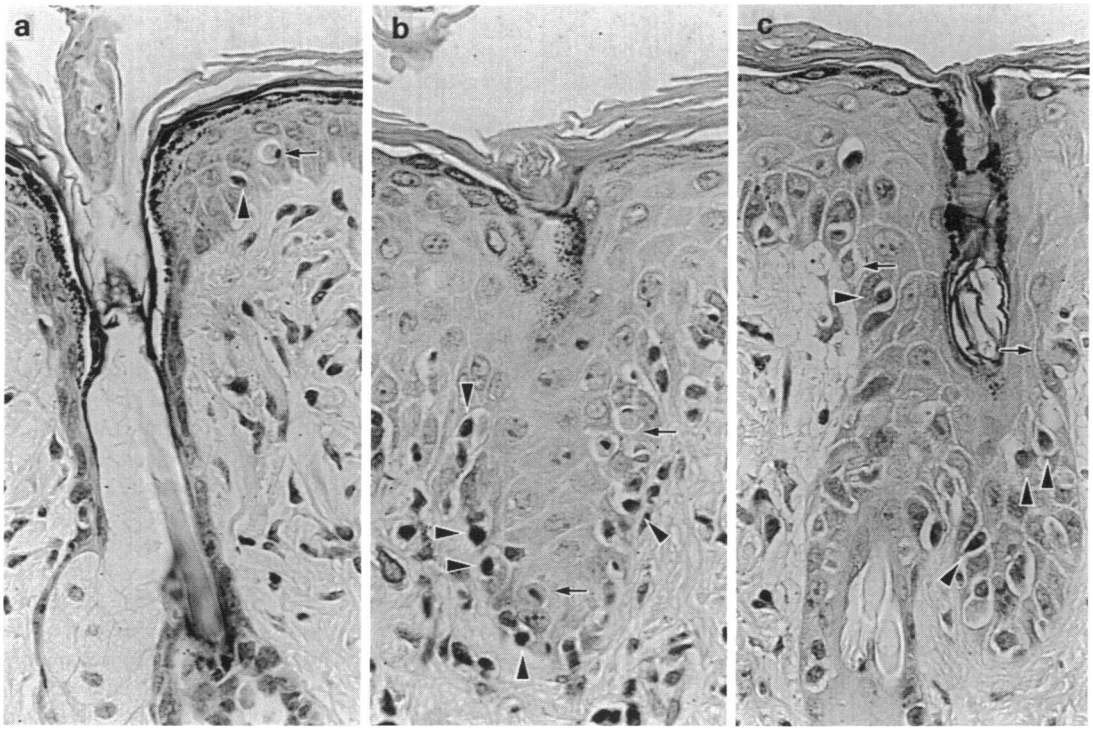

Fig. 1. Histological findings of the skin lesion of: (a) (B6 × bm1)F1; (b) (B6 × bm12)F1; and (c) (bm1 × bm12)F1 mice 6 days after local injection of B6 whole T cells. The epidermis shows thickening and many mononuclear cells (arrowheads) are present in the intracellular spaces of the lower epidermis in (b) and (c). Some epidermal cells become eosinophilic (arrows). These histologic changes are very mild in (a). Haematoxylin–eosin staining. Magnification ×430.

necrosis was seen. Culture medium-injected skin appeared histologically normal (data not shown).

Histological changes in the skin of MHC class I-disparate recipient mice

When B6 cells were injected into unirradiated (B6 × bm1)F1 mice, histological changes of cutaneous GVHD were very mild as compared with those seen in (B6 × bm12)F1 recipient mice (Fig. 1a). The epidermis showed mild thickening, and a small number of intraepidermal mononuclear cells around foci of epidermal cell necrosis was observed. Injected donor cells in the dermis decreased in number more rapidly than those in (B6 × bm12)F1 recipient mice and cutaneous lesions disappeared by 6–7 days after injection.

Injection of purified $CD4^+$ donor cells did not induce epidermal cell necrosis, whereas injection of purified $CD8^+$ cells

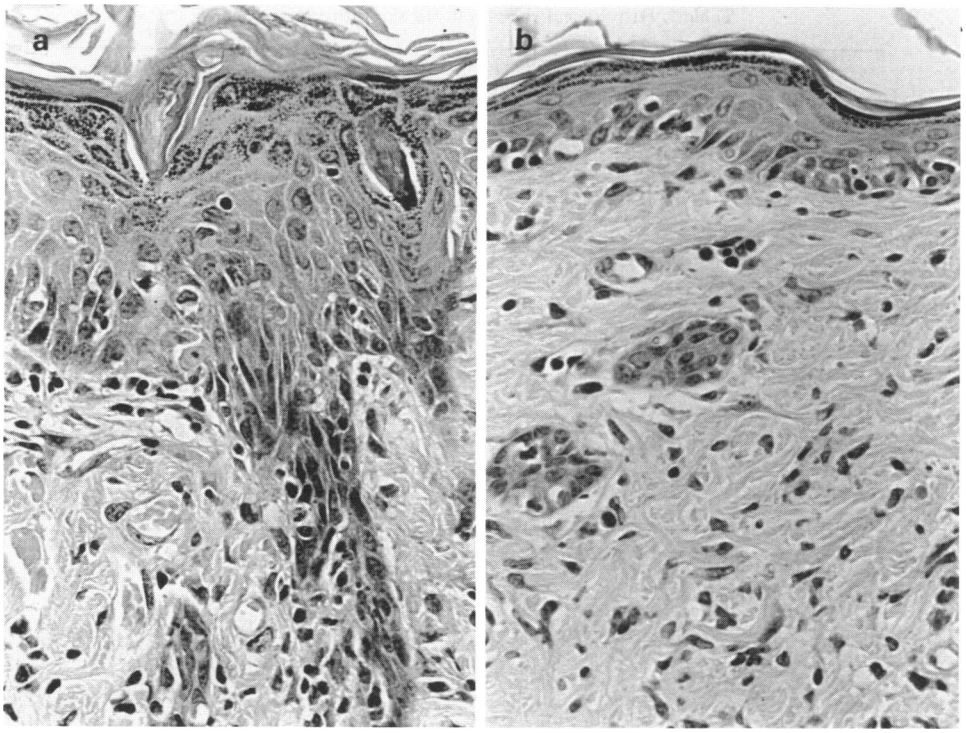

Fig. 2. Cutaneous GVHD lesions of (B6 × bm12)F1 mice 5 days after local injection of B6 $CD4^+$ (a) or $CD8^+$ (b) cells. Haematoxylin–eosin staining. Magnification × 360.

Table 2. Staining pattern of intraepidermal cellular infiltrates

Donor cell preparation	$CD4^+$	$CD8^+$
Whole T cells	++	++
$CD4^+$ cells	++	+
$CD8^+$ cells	±	++

B6 donor cells (1×10^7) were injected into (B6 × bm12)F1 or (bm1 × bm12)F1 recipient mice. There was no difference in the staining pattern between these two recipients.

The mean number of positive cells in the epidermis are expressed with the following symbols: ±, <5; +, 5–9; and ++, >9 cells per linear mm of epidermis.

induced weak cutaneous GVHD characterized by mild thickening of the epidermis and a few intra-epidermal mononuclear cells around foci of epidermal cell necrosis as seen in case of whole T cell injection.

Histological changes in the skin of MHC class I+II-disparate recipient mice

Histological changes observed in the skin of unirradiated (bm1 × bm12)F1 mice after B6 whole T cell injection were essentially identical to those seen in (B6 × bm12)F1 recipient mice (Fig. 1c). Typical lesions of cutaneous GVHD could be induced also after purified $CD4^+$ or $CD8^+$ donor cell injection.

Immunohistochemical studies on the intraepidermal cellular infiltrates

The subsets of intra-epidermal cellular infiltrates in the skin of cutaneous GVHD were analysed immunohistochemically. Since intraepidermal cellular infiltrates in (B6 × bm1)F1 recipient mice were very few, it was difficult to analyse their subsets quantitatively. However, when whole T or $CD8^+$ cells were injected, the great majority of the infiltrating cells seemed to be $CD8^+$ in (B6 × bm1)F1 mice (data not shown).

There was no significant difference in the staining pattern between (B6 × bm12)F1 and (bm1 × 12)F1 recipient mice during 4–6 days after injection. The staining pattern of intraepidermal cellular infiltrates 5 days after injection is summarized in Table 2. In the lesions of cutaneous GVHD caused by whole T cells, both $CD4^+$ and $CD8^+$ cells appeared in the epidermis (Fig. 3a, b). When $CD4^+$ cells were injected, $CD4^+$ cells were dominant in the intra-epidermal infiltrates, and a small number of $CD8^+$ cells that may be of recipient origin were also found in the epidermis (Fig. 3c, d). However, in mice with $CD8^+$ cell injection, most of the infiltrating cells were $CD8^+$, and $CD4^+$ cells were rarely seen in the epidermis (Fig. 3e, f).

To determine the origin of intra-epidermal infiltrates, T cells from B10.Thy-1.1 mice, which carries different Thy-1 antigen (Thy-1.1) from (B6 × bm12)F1 and (bm1 × bm12)F1 recipient mice (Thy-1.2), were used as donor cells instead of B6 T cells. When T cells from B10.Thy-1.1 mice were injected into (B6 × bm12)F1 or (bm12 × bm12)F1 recipients, typical cutaneous GVHD were also observed during 4–6 days after injection and immunohistochemical studies revealed that the great

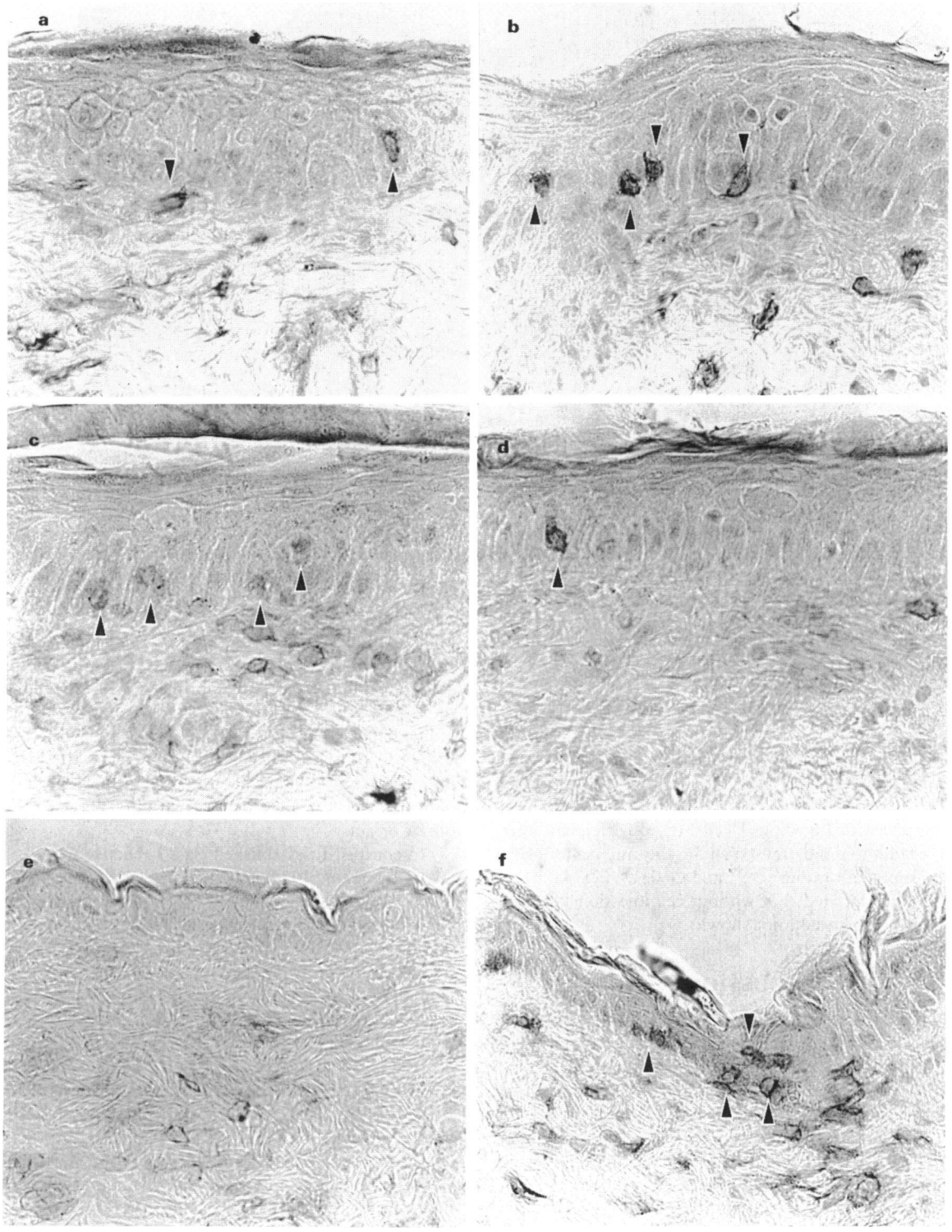

Fig. 3. Immunoperoxidase staining of cutaneous GVHD lesions of (B6 × bm12)F1 mice 5 days after local injection. (a) and (b), Whole T cell injection; (c) and (d), $CD4^+$ cell injection; (e) and (f), $CD8^+$ cell injection. Sections were stained with anti-CD4 MoAb (a, c, e) or anti-CD8 MoAb (b, d, f), and positive cells are shown by arrowheads. Magnification × 480.

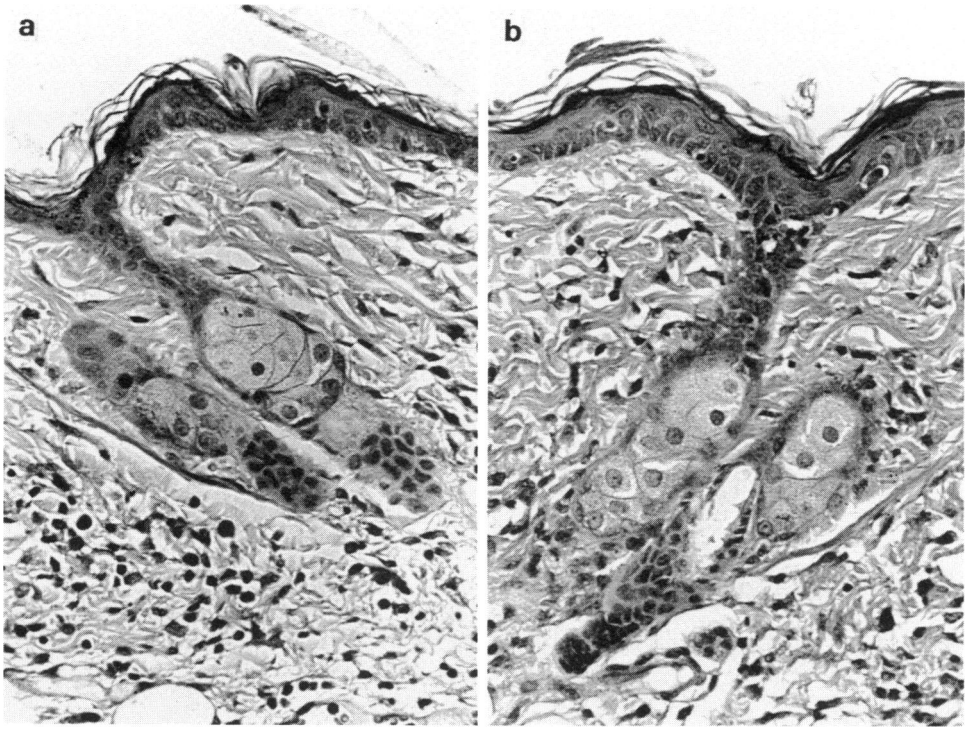

Fig. 4. Cutaneous GVHD lesions of sublethally irradiated (B6 × bm1)F1 (a) and (B6 × bm12)F1 (b) mice 5 days after local injection of B6 whole T cells. In both recipients, sublethal irradiation prevents the development of cutaneous lesions (compare with Fig. 1 a, b). Magnification × 290.

majority of intraepidermal T cells were of donor origin (Thy-1.1$^+$, data not shown).

Histological changes in the skin of sublethally irradiated recipient mice

To determine the effect of sublethal irradiation on the development of cutaneous GVHD, (B6 × bm1)F1 and (B6 × bm12)F1 recipient mice were irradiated at a dose of 4 Gy and subsequently injected with B6 whole T cells. In both recipient mice, sublethal irradiation did not result in the augmentation of cutaneous lesions but rather prevented GVHD (Fig. 4a, b).

Sublethally irradiated skin without cell injection appeared histologically normal (data not shown).

DISCUSSION

Intravenous injection of unprimed T cells into unirradiated semi-allogeneic recipient mice results in acute lethal GVHD only when recipients are disparate to donors at both MHC class I and II loci (Rolink, Pals & Gleichmann, 1983). Although the skin is a major target organ in human GVHD after bone-marrow transplantation (Glucksberg *et al.*, 1974), cutaneous lesions rarely occur in the murine systemic GVHD system without irradiation (our unpublished observation). For an alternative approach to induce cutaneous GVHD in unirradiated recipients, we described previously (Watanabe *et al.*, 1985, 1989) the cutaneous GVHD model induced by local injection of alloreactive T cell clones. Using this system, we showed that both MHC class I- and II-reactive T cell clones can induce typical cutaneous lesions of GVHD after local injection into unirradiated or sublethally irradiated recipients. However, it has remained unknown whether freshly prepared T cells are able to induce cutaneous GVHD after local injection into unirradiated recipients in single MHC disparity. In the present study, we found that 1×10^7 unprimed B6 T cells and their CD4$^+$ or CD8$^+$ subset were able to induce cutaneous GVHD in unirradiated MHC class II-disparate (B6 × bm12)F1 and I + II-disparate (bm1 × bm12)F1 mice 4–6 days after intradermal injection. Analysis of the phenotype of intraepidermal infiltrates indicated that the great majority of infiltrating T cells were of donor origin.

In contrast to established class I-reactive CD8$^+$ cell clones (Watanabe *et al.*, 1985), freshly prepared B6 T cells elicited very mild histological changes in MHC class I-disparate (B6 × bm1)F1 recipient mice. The mechanism behind this finding is unknown. However, as mentioned above, similar findings have been reported in systemic acute GVHD model (summarized in Table 3). Although i.v. injection of T cells into irradiated MHC class I- and/or II-disparate recipients results in acute lethal GVHD (Korngold & Sprent, 1985, 1987; Sprent *et al.*, 1986, 1988), in the case of unirradiated recipients, acute GVHD occurs only in certain strain combinations of MHC class I + II disparity (Rolink *et al.*, 1983). It has been reported (Liu, Skeen & Fritz, 1990) that even in the parent-into-F1 systems, host anti-donor responses might occur and that their extents vary with the combinations of donor and host strain. This and the above findings in systemic acute GVHD raise the possibility that radiosensitive host anti-donor responses might prevent the development of cutaneous lesions especially with MHC class I-disparate combination in our local GVHD model. However, such explanation would be less likely because sublethal irradiation of class I-disparate recipients did not result in the augmentation of cutaneous lesions. It was also demonstrated (Murphy *et al.*, 1990) that radioresistant anti-donor responses

Table 3. Comparison of local cutaneous GVHD and systemic acute GVHD

Type of GVHD	Disparity		
	MHC class I	MHC class II	MHC class I+II
Cloned T cell-induced local cutaneous GVHD	++*	++	Not done
Unprimed T cell-induced local cutaneous GVHD	+*	++	++
Unprimed T cell-induced systemic acute GVHD	−†	−	+

Three models of murine GVHD induced in unirradiated recipients using B6 mutant strains are summarized (Rolink *et al.*, 1983; Watanabe *et al.*, 1985, 1989).

* Estimation of local cutaneous GVHD was done by the existence of epidermal cell necrosis (see legend of Table 1).

† Estimation of systemic acute GVHD was done by the mortality.

mediated by NK cells and radioresistant T cells are generated in parent-into-F1 systems. The possibility that such radioresistant cells are generated and prevent the development of MHC class I-disparate cutaneous GVHD remains to be elucidated in our model. It should be noted that sublethal irradiation somewhat suppress both MHC class I- and II-disparate cutaneous GVHD. This finding suggests that an important host component such as alloantigen-presenting cells might also be impaired by irradiation (Stegall *et al.*, 1990).

Another possible explanation for the difference in GVHD-inducing capacity between unprimed T cells and T cell clones is that low frequency of anti-MHC class I repertoire in unprimed T cells might cause them to be unable to induce severe cutaneous lesions in MHC class I-disparate recipients. There have been several lines of evidence which support this concept. Since alloreactive T cell clones contain high frequency of responding cells to the relevant MHC loci, both MHC class I- and II-reactive T cell clones can induce severe cutaneous GVHD after intradermal injection of small numbers ($3–5 \times 10^6$) (Watanabe *et al.*, 1985, 1989) as compared with unprimed T cells (1×10^7). This is the case also in systemic acute GVHD. To induce acute lethal GVHD in unirradiated recipients, certain strain combinations of MHC class I+II-disparity and relatively large numbers (10^8) of donor cells are required (Rolink *et al.*, 1983). Recently, Lehmann *et al.* (1990) demonstrated that alloreactive T cell clones can induce acute lethal GVHD in unirradiated recipients with single MHC disparity after i.v. injection of $5 \times 10^6–10^7$ cells. The conclusion from these findings is that large numbers of donor cells are required for the induction of GVHD in unirradiated recipients with single MHC disparity and therefore only established clones, which have high frequency of alloreactive T cells, but not unprimed T cells, are able to induce GVHD in certain strain combinations.

Another interesting finding is that freshly prepared $CD8^+$ cells alone could induce cutaneous GVHD in MHC class II-disparate recipients. Immunohistochemical examinations revealed that the great majority of intraepidermal infiltrates were $CD8^+$, and $CD4^+$ cells were rarely seen in the cutaneous lesions. Although it seems a well established idea that $CD8^+$ cells selectively respond to MHC class I antigens (review by Swain, 1983), there have been several reports of MHC class II-reactive $CD8^+$ T cell clones (Haas & von Boehmer, 1984; Golding & Singer, 1985; Shinohara & Kojima, 1984). The functions of MHC class II-reactive $CD8^+$ cells *in vivo* are still unknown. Previously, we found that MHC class II-reactive $CD8^+$ T cell clones were able to induce cutaneous GVHD in MHC class II-disparate recipient mice, when injected intradermally (Watanabe *et al.*, 1989). The findings obtained in this study support the concept that $CD8^+$ cytotoxic T cells responding to allogeneic class II antigens *per se* elicited cutaneous GVHD in MHC class II-disparate recipients, although 'help' by the contaminating $CD4^+$ cells could not be completely denied. Cutaneous lesions induced by unprimed $CD8^+$ cell injection into MHC class II- or I+II-disparate recipients showed relatively mild thickening of the epidermis as compared with those by $CD4^+$ cell injection. This is consistent with previous findings (Piguet *et al.*, 1987) that $CD4^+$ cells play a major role in thickening of the epidermis observed in class II-disparate GVHD.

The present study has revealed that unprimed T cells are able to induce severe cutaneous GVHD in unirradiated recipient mice after local injection only in MHC class II and I+II disparity. It was suggested that radioresistant host anti-donor responses and/or low frequency of anti-MHC class I repertoire in unprimed T cells might be responsible for the failure in induction of MHC class I-disparate GVHD. This experimental system provides a useful model for analysis of the regulation mechanisms in the induction of GVHD by unprimed T cells.

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